·Basic Research·

Effect of brain-derived neurotrophic factor on c-jun expression in the *rd* mouse retina

Rui Chen, Xiao-Bei Yin, Chun-Xia Peng, Gen-Lin Li

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Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Key Laboratory of Ophthalmology and Visual Sciences, Beijing 100730, China

Correspondence to: Gen-Lin Li. Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Key Laboratory of Ophthalmology and Visual Sciences,Beijing 100730,China.ligenlin@hotmail.com

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Abstract

• AIM: To determine the location of c-jun protein, dynamic changes in c-*jun* mRNA and protein expression, and ultrastructure characteristics in the *rd* mouse retina, following a single dose of brain-derived neurotrophic factor (BDNF) in a short period of time.

• METHODS: A single intravitreal injection of BDNF at two dosages (25μ g/L or 50μ g/L) was given to the right eye of the *rd* mouse at age 2 and 3 weeks respectively. Two weeks after injection, the location of c-jun protein in the retina was observed by immunofluorescence detection, c-*jun* mRNA and protein expression in retinas were detected by quantitative real time polymerase chain reaction (RT-PCR) and western immunoblotting analysis, ultrastructure characteristics of retinas were detected by transmission electron microscope (TEM) observation.

• RESULTS: c-jun protein was expressed in the inner nuclear layer (INL) of retina. BDNF at two dosages (25μ g/L and 50μ g/L) increased c-*jun* mRNA expression at PN-4 weeks respectively (P_1=0.019, P_2=0.021). 50μ g/L BDNF increased c-jun protein expression at PN-4 weeks (P=0.000). The retinal ultrastructure was improved.

• CONCLUSION: The effects of BDNF exerts on the c-jun expression in the retina are dose-dependent and time-dependent, which may mediate photoreceptor rescue indirectly in the pathological process of retinitis pigmentosa (RP)at early stage.

• KEYWORDS: brain-derived neurotrophic factor; c-jun; ERK; photoreceptor

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INTRODUCTION

Retinitis pigmentosa (RP) is one of the most common inherited retinal degenerative disorders, which are currently the leading cause of incurable blindness. It is a group of inherited retinal dystrophies characterized by progressive photoreceptor degeneration [1,2]. Brain-derived neurotrophic factor (BDNF), an important neuroprotective factor that belongs to the neurotrophin family, exerts its survival-promoting effects on retinal photoreceptors and is critically involved in a variety of retinal degenerative animal models [3,4]. The BDNF effects are known to be mediated by TrkB receptor-induced activation of two key signaling pathways including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3-K)/Akt[5].

c-jun, an important member of the jun protein family [6], constitutes the activator protein (AP)-1 transcription factor complex with c-fos and is involved in many biological processes, including cell differentiation, proliferation, apoptosis, and survival[7-9]. Several studies have reported the correlation between BDNF and c-jun. BDNF and c-jun may be simultaneously induced in cortical impact brain injury [10] and spinal cord injury [11]. Recent studies have revealed that BDNF mediated ERK1/2 phosphorylation and c-jun induction and then induced sulfiredoxin against 3-NP toxicity in primary rat cortical neurons, namely "BDNF → ERK1/2-Pi → c-jun → sulfiredoxin → 3-NP resistance". This result established that c-jun mediated the BDNF-dependent neuroprotective effects directly [12]. These results raises the question that whether the BDNF-dependent neuroprotective effects are related to c-jun in the pathological process of RP.

In the present study, we observed the effects of BDNF on dynamic changes in c-*jun* mRNA and protein expression in the *rd* mouse retina.

MATERIALS AND METHODS

Materials All the intravitreal injections were performed by one of the authors (NHVC). *rd* mice(At age 2 and 3 weeks, weighing 7-10g) were anesthetized by 5% chloral hydrate (8μg/L). 2μL of BDNF recombinant protein [50μg/L or

100μg/L, diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA),PeproTech, USA] (32 *rd* mice)or PBS (PBS controls, 16 *rd* mice) were injected into the vitreous chamber of the left eye using a 10μL Hamilton syringe adapted with a 29 gauge needle. Contralateral eyes or unoperated eyes served as intact controls (*rd* controls). The needle tip was inserted into the superior hemisphere of the eye, at the level of the pars plana, at a 45° angle through the sclera into the vitreous body. The injection was performed within 1 minute and the needle was kept in place for an additional period of 2 minutes, after which it was gently removed. 16 age-matched C57BL/6J mice with no intravitreal injections were used as blank controls. Two weeks after injection, eyes were enucleated for the following detection. All animals used in this study were cared for and handled according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Methods

Immunohistochemistry Eyes were embedded with optimal cutting temperature (OCT) and serial 7μm of section were produced. Sections were fixed with 4% paraffin for 10 minutes, rinsed with 0.1 mol/L PBS (pH 7.4, 3×5 minutes) and incubated in 5% BSA for 20 minutes. rabbit polyclonal anti-c-jun antibody (1:100, Abcam, USA)incubation was performed in PBS overnight at 4℃ . Slices were then incubated with fluoresceinisothiocyanate-conjugated secondary antibody for 45 minutes at 37℃ , rinsed with 0.05mol/L PBS (3×5 minutes), Sections with PBS instead of primary antibody were included as negative controls. I and viewed with a Fluorescence images were acquired by fluorescence microscope (Leica DM4000B,Germany)and analyzed by Image Pro Plus 6.0 software (MC, USA).

RNA isolation and quantitative RT-PCR Retinas were removed through a slit in the cornea and immediately frozen in liquid nitrogen. RNA was isolated using Trizol reagent (Invitrogen), and cDNA was synthesized with 2μg of total RNA using the Transcript First-Strand cDNA Synthesis Kit (Transgene Biotechnology Inc., Beijing, China). Quantitative real-time PCR was performed using the UltraSYBR mixture (Cwbiotech, China) in the Stratagene Mx3000P™ Real-Time PCR System (Agilent Technologies, CA). PCR primers for mouse c-jun were forward: 5'- TTCT ACGACGATGCCCTCAAC -3' and reverse: 5'- CAGGTTC AAGGTCATGCTCTGTT -3'. PCR primers for mouse GAPDH were forward: 5'- CGACTTCAACAGCGACAC TCAC -3' and reverse: 5'- CCCTGTTGCTGTAGCCAAAT TC -3'. The thermal cycling conditions were as follows: initial denaturation at 95℃ for 10 minutes, followed by 40 cycles of denaturing at 95℃ for 15 seconds, annealing at 60℃ for 30 senconds and extension at 72℃ for 30 seconds. Data were normalized according to copy number of GAPDH mRNA and were analyzed by SDS 2.2 software (ABI, USA). All experiments were performed in triplicate.

Western immunoblotting Retinas were removed through a slit in the cornea and immediately frozen in liquid nitrogen. Retinal protein was extracted with lysis buffer (Beyotime, China) on ice for 30 minutes and was then centrifuged at 15 000r/min at 4℃ for 15 minutes. Protein concentrations were determined using the BCA method. An aliquot of this total protein were boiled for 4 minutes, resolved by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 at room temperature for 1 hour, and then incubated with mouse monoclonal anti-α-tubulin (1:1000, Santa Cruz, USA) or rabbit polyclonal anti-c-jun (1:1000, Abcam, USA) antibodies at 4℃ overnight, followed by an incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:10 000,Cwbiotech, China) at room temperature for 1 hour. The chemiluminescence reaction was performed using ECL reagent (Thermo Scientific). Protein bands were scanned and evaluated by densitometry using Quantity One analysis software (Bio-Rad, USA), which was normalized for α-tubulin density. All experiments were performed in triplicate.

Electron microscopy The enucleated eyes were dissected along the equators, and the retina was incubated for 4 hours in 2.5% glutaraldehyde (in 100mmol/L phosphate buffer, pH 7.4). The retina was postfixed in 1% buffered osmium tetroxide and dehydrated using graded ethanol solutions. Semi-thin sections (1μm) were prepared, stained with toluidine blue and examined by light microscopy. Ultrathin sections (0.5μm) of selected areas were then prepared and stained with uranyl acetate and lead citrate for transmission electron microscope (H 7650TEM; Hitachi, Tokyo, Japan).

Statistical Analysis Data were represented as mean±SD. Statistical analysis of the data was performed by SPSS18.0 using one-way analysis of variance (ANOVA) for planned comparisons among the various treatments at the same point and a least significant difference-*t* (LSD-*t*) test for planned comparisons between the same treatments at different time point. A *P*-value less than 0.05 was considered significant.

RESULTS

Immunohistochemical location of c-jun expression in the retina c-jun protein was only expressed in the INL at age 4 and 5 weeks. At age 4 weeks, c-jun protein levels increased in the 25μg/L BDNF group compared with the *rd* control group(P=0.212) and the PBS group(P=0.020), but decreased compared with the blank control group(P=0.001). 50μg/L BDNF treatment elevated c-jun protein expression compared with the *rd* control group (P=0.000), the PBS group (P=0.000), the blank control group (P=0.293), and the 25μg/L BDNF group (P=0.000, Figure 1).

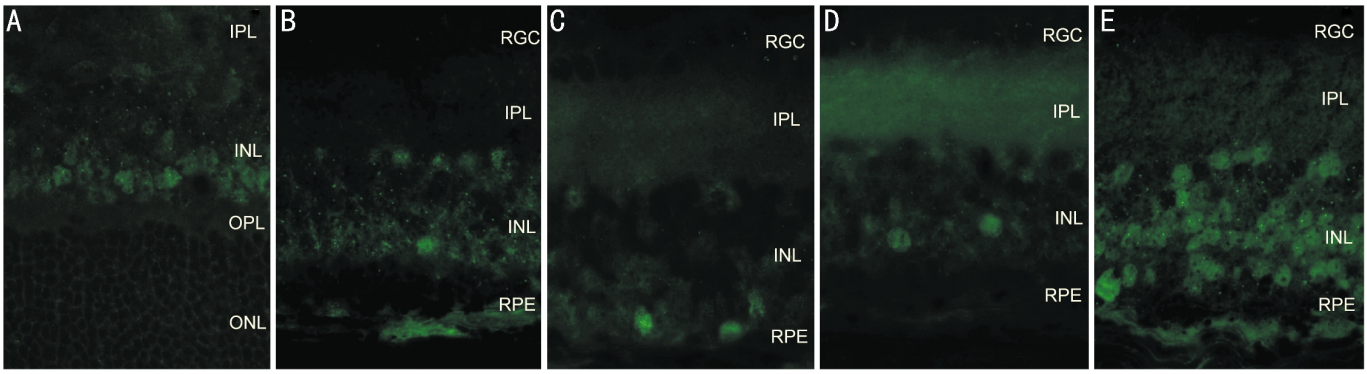

Figure 1 Immunohistochemical location of c-jun expression in the retina at age 4 weeks(×400) A: the Blank control group; B: the *rd* control group; C: the PBS control group; D: the 25μg/L BDNF group; E: the 50μg/L BDNF group. (RPE: retinal pigment epithelium; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer).

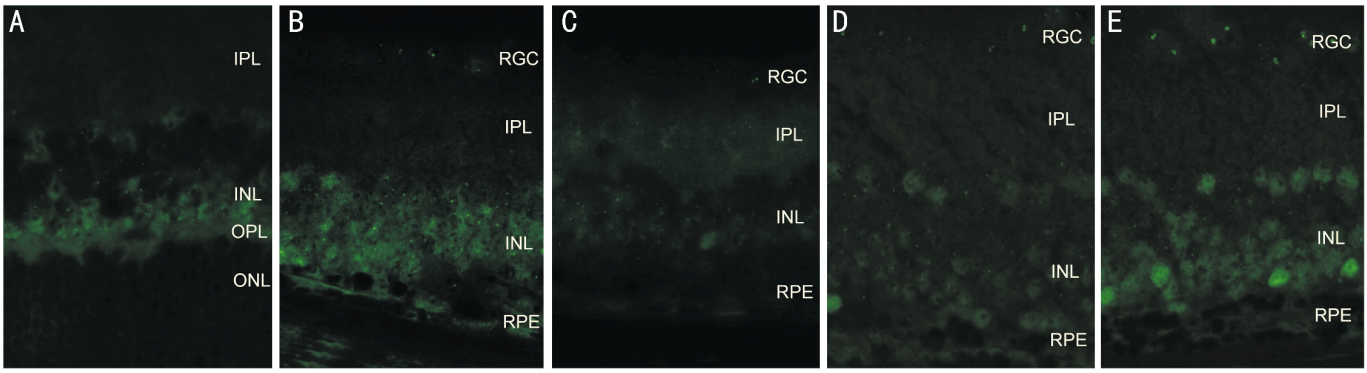

Figure 2 Immunohistochemical location of c-jun expression in the retina at age 5 weeks (×400) A: the Blank control group; B: the *rd* control group; C: the PBS control group; D: the 25μg/L BDNF group; E: the 50μg/L BDNF group.

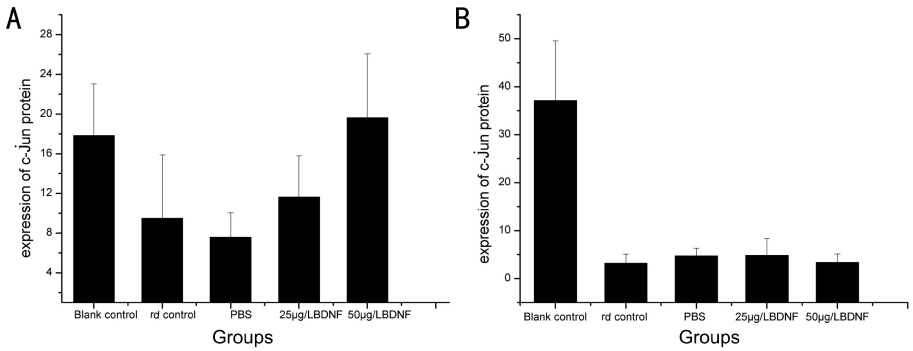

Figure 3 Immunohistochemical location of c-jun expression in the retina A: groups at the age 4 weeks; B: groups at age 5 weeks.

Table 1 c-jun protein expression in the retina by immunohistochemistry (mean±SD)

	C57BL/6	*rd*	PBS	25μg/L BDNF	50μg/L BDNF
PN-4w	17.84±5.20	9.52±6.38	7.60±2.45	11.67±4.12	19.65±6.42
PN-5w	37.14±12.40	3.17±1.94	4.75±1.60	4.87±3.45	3.36±1.80

At age 5 weeks, c-jun protein levels increased in the 25μg/L BDNF group compared with the *rd* control group(P=0.571), but decreased compared with the PBS group (P=1.000) and the blank control group (P=0.000). In the 50μg/L BDNF group, c-jun protein expression increased compared with the rd control group (P=1.000) and the PBS group (P=0.177), but decreased compared with the blank control group (P = 0.000), and the 25μg/L BDNF group (P =0.695, Figure 2, Table 1).

At age 5 weeks, c-jun protein expression decreased in the *rd* control group (P=0.000), the PBS group (P=0.000), and the BDNF group (P_1=0.000, P_2=0.000), but increased in the blank control group compared with the same group respectively at age 4 weeks (P=0.000, Figure 3).

BDNF results in dynamic changes in c-*jun* mRNA expression in the retina At age 4 weeks, BDNF treatment (25μg/L and 50μg/L) upregulated c-*jun* mRNA expression compared with the *rd* control group (P_1=0.019, P_2=0.021), the PBS group (P_1=0.550, P_2=0.596), the blank control group (P_1=0.024, P_2 =0.027). c-*jun* mRNA expression was similar between the BDNF groups.

At age 5 weeks, BDNF treatment slightly upregulated c-*jun*

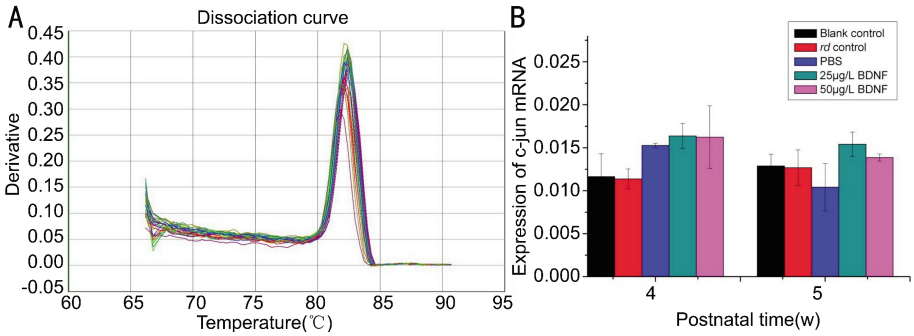

Figure 4 Dynamic changes in c-jun mRNA expression in the retina A: dissociation curve of c-jun produced by real-time polymerase chain reaction; B: altered c-jun mRNA expression.

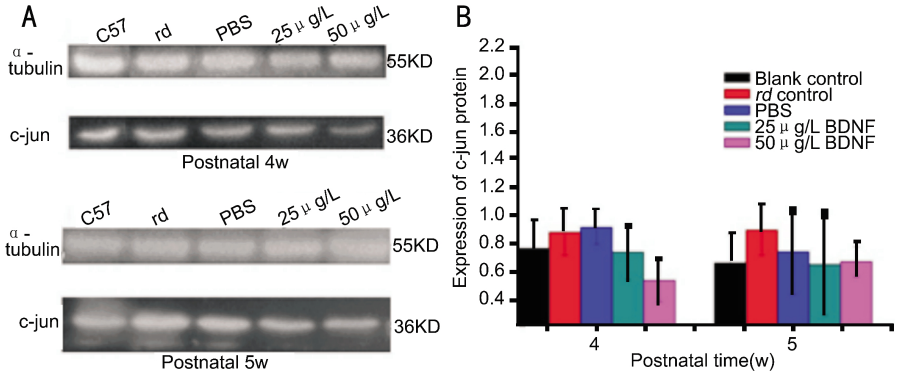

Figure 5 Dynamic changes in c-jun protein expression in the retina A: electrophoresis of c-jun protein expression; B: altered c-Jun protein expression.

Table 2 c-jun mRNA expression in the retina (mean±SD)

	C57BL/6	*rd*	PBS	25μg/L BDNF	50μg/L BDNF
PN-4w	0.0116±0.0026	0.0114±0.0011	0.0153±0.0003	0.0164±0.0015	0.0163±0.0037
PN-5w	0.0129±0.0014	0.0127±0.0021	0.0104±0.0028	0.0154±0.0014	0.0139±0.0004

Table 3 c-jun protein expression in the retina (mean±SD)

	C57BL/6	*rd*	PBS	25μg/L BDNF	50μg/L BDNF
PN-4w	0.77±0.20	0.89±0.16	0.92±0.12	0.74±0.20	0.54±0.15
PN-5w	0.67±0.21	0.90±0.18	0.75±0.31	0.66±0.36	0.68±0.11

mRNA expression compared with the *rd* control group, the PBS group, and the blank control group. c-*jun* mRNA expression in the 50μg/L BDNF group decreased compared with the 25μg/L BDNF group (P=0.065, Table 2).

At age 5 weeks, c-*jun* mRNA expression gradually increased in the *rd* control group (P=0.391) and the blank control group (P=0.507), but decreased in the PBS group(P=0.039) and the 50μg/L BDNF group (P=0.323) compared with the same group respectively at age 4 weeks. There were no obvious differences between the two time points in the 25μg/L BDNF group (P=0.458, Figure 4).

BDNF results in dynamic changes in c-jun protein expression in the retina At age 4 and 5 weeks, BDNF treatment decreased c-*jun* protein expression compared with the *rd* control group and the PBS group, but similar to the blank control group. c-*jun* mRNA expression was similar between BDNF groups. c-*jun* protein levels in the 50μg/L BDNF group was lower than the 25μg/L BDNF at age 4 weeks and similar to the 25μg/L BDNF group at age 5 weeks((P_1=0.134, P_2=0.731).

At age 5 weeks, c-*jun* mRNA expression gradually decreased in the blank control group (P=0.571), the PBS group (P=0.41), and the 25μg/L BDNF group (P=0.737), but increased in the *rd* group (P=0.912) and the 50μg/L BDNF group (P=0.283) compared with the same group respectively at age 4 weeks (Figure 5, Table 3).

BDNF results in changes in retinal ultrastructure At age 4 and 5 weeks, BDNF treatment improved the retinal ultrastructure.vacuolization and destruction of plasma and nuclear membranes were improved in the retinal pigment epithelium (RPE). In the outer nuclear layer (ONL), the number of nuclei increased and the swollen cell bodies and the damaged mitochondria were improved. The inner segments of cones were occasionly seen. The arrays of microtubules and synaptic structures were improved in the outer plexiform layer (OPL).There were more and larger

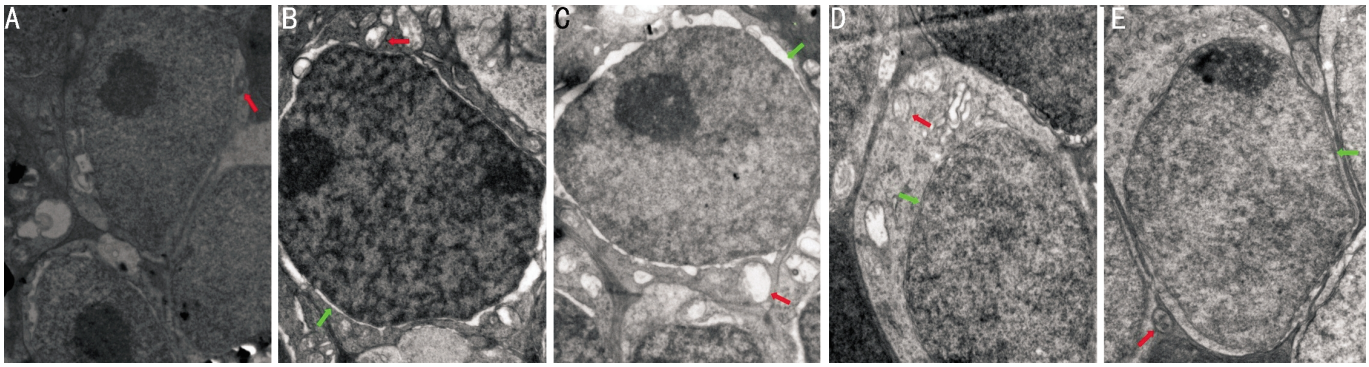

Figure 6 Changes in retinal ultrastructure at age 4 weeks A: the Blank control group(×15 000); B: the *rd* control group(×25 000); C: the PBS control group(×20 000); D: the 25μg/L BDNF group (×25 000); E: the 50μg/L BDNF group(×25 000). (Red arrows: mitochondria, green arrows: nuclear membranes).

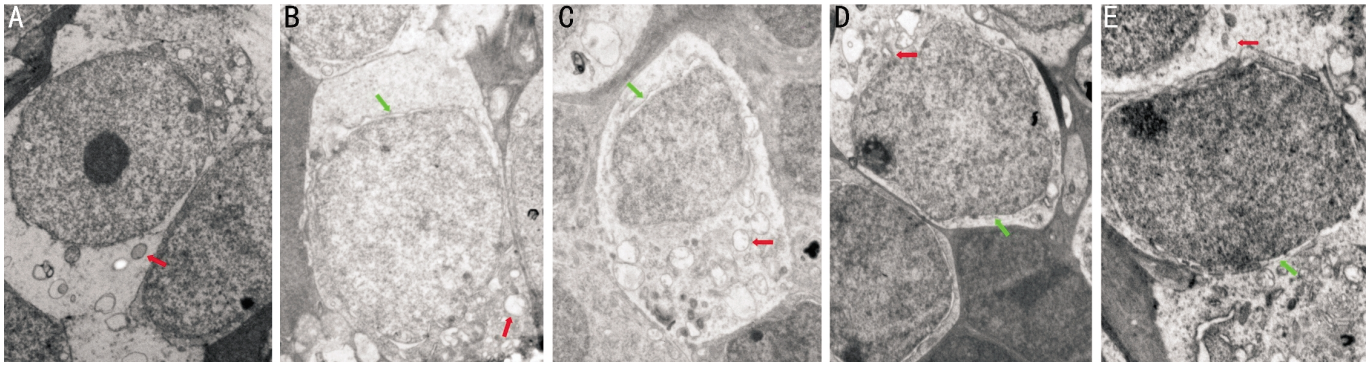

Figure 7 Changes in retinal ultrastructure at age 5 weeks A: the Blank control group (×15 000); B: the *rd* control group (×15 000); C: the PBS control group (×12 000); D: the 25μg/L BDNF group (×15 000); E: the 50μg/L BDNF group (×20 000). (Red arrows: mitochondria, green arrows: nuclear membranes).

mitochondria and ribosomes with fine morphology in the plasma and destruction of plasma and nuclear membranes were improved in the INL. There were no obvious changes in the inner plexiform layer and ganglion cell layer (Figure 6 and 7).

DISCUSSION

In the present study, a single dose of BDNF (25μg/L and 50μg/L) treatment prominently enhanced c-*jun* mRNA levels at age 4 weeks and the levels were greater in the 50μg/L BDNF group compared with the 25μg/L BDNF group. Corresponding c-jun protein changes at the same time point were observed following modification of c-jun gene expression shown by immunohistochemistry. Meanwhile, there were no obvious changes of c-*jun* mRNA and protein expression at age 5 weeks. These results suggested that The effects of BDNF exerts on the c-jun expression in the retina were dose-dependent and time-dependent and may be involved in the pathological process of RP at early stage.

The effects of c-jun on neuronal cells are controversial. c-jun has been reported to be involved in neuronal survival and axon regeneration in adults [13]. c-jun has been hypothesized to act as a death signal in degenerating neurons and may play a role in photoreceptor apoptosis[14,15]. These contradictory results may be related to the various signaling pathways, which results in the mediation of distinct biological actions [16,17]. c-jun can be phosphorylated directly by activated JNK, ERK1/2, and p38 cascades. p38 and JNK has been shown to be important signal transduction pathways contributing to glia-induced neuron death, but ERK pathway was not involved in neuron loss[18]. JNK/c-jun could directly inhibit expression of Nrl, which is essential for rod differentiation and function [19]. ERK pathway could be activated to product AP-1, which was a prosurvival signal in mediating photoreceptor cell death [20]. Recent studies have revealed that BDNF mediated ERK1/2 phosphorylation and c-jun induction and then induced sulfiredoxin against 3-NP toxicity in primary rat cortical neurons[12].

Studies have shown that photoreceptor cells did not express BDNF receptors [21] and BDNF had no direct effect on isolated photoreceptor cells [22]. BDNF binded to TrkB receptor in Müller cells and was involved in photoreceptor rescue indirectly [23]. After a diverse array of retinal injuries, Müller cell reactivity was thought to promote initially a survival response through the release of neurotrophic factors and antioxidants [24-26] and this early nonspecific responses involved ERK activation and upregulation of GFAP expression [27]. BDNF has been shown that it can modulate production and release of several neutrophins from Müller

cells during light-induced retinal degeneration to influence photoreceptor survival indirectly [28]. Intraocular administration of BDNF can activate ERK pathway in Müller glial cells [29]. In the present study, c-jun protein was expressed in INL, may be in Müller cells and the retinal ultrastructure was improved. It has been established that receptor isoforms of BDNF were expressed temporally and spatially on Müller cells during light-induced retinal degeneration [30]. In the present study, c-jun expression elevation mediated by BDNF was at the earlier time point. These results suggest that BDNF may bind to TrkB receptor in Müller cells and activate ERK pathway to produce c-jun, which mediates photoreceptor rescue indirectly in the pathological process of RP at early stage.

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